EXHIBIT A

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non-homologous recombination, and allowing over-expression of the endogenous gene in the cell.

The cell containing the vector is screened for expression of the gene.

The cell over-expressing the gene can be cultured *in vitro* so as to produce desirable amounts of the gene product of the endogenous gene whose expression has been activated or increased. The gene product can then be isolated and purified.

Alternatively, the cell can be allowed to express the desired gene product *in vivo*.

The vector construct can consist essentially of the transcriptional regulatory sequence and the splice donor sequence.

Any of the vector constructs used in the methods described herein can also include a secretion signal sequence. The secretion signal sequence is arranged in the construct so that it will be operably linked to the activated endogenous protein. Thereby, secretion of the protein of interest occurs in the cell, and purification of that protein is facilitated. Accordingly, methods can include a step in which the protein expression product is secreted from the cell.

The invention also encompasses cells made by any of the above methods. The invention encompasses cells containing the vector constructs, cells in which the vector constructs have integrated, and cells which are over-expressing desired gene products from an endogenous gene, over-expression being driven by the introduced transcriptional regulatory sequence.

The cells can be isolated and cloned.

The methods can be carried out in any cell of eukaryotic origin, such as fungal, plant or animal. Preferred embodiments include vertebrates and particularly mammals, and more particularly, humans.

A single cell made by the methods described above can over-express a single gene or more than one gene. More than one gene in a cell can be activated by the integration of a single type of construct into multiple locations in the genome. Similarly, more than one gene in a cell can be activated by the

integration of multiple constructs (i.e., more than one type of construct) into multiple locations in the genome. Therefore, a cell can contain only one type of vector construct or different types of constructs, each capable of activating an endogenous gene.

The invention is also directed to methods for making the cells described above by one or more of the following: introducing one or more of the vector constructs; allowing the introduced construct(s) to integrate into the genome of the cell by non-homologous recombination; allowing over-expression of one or more endogenous genes in the cell; and isolating and cloning the cell.

The invention also encompasses methods for using the cells described above to over-express a gene that has been characterized (for example, sequenced), uncharacterized (for example, a gene whose function is known but which has not been cloned or sequenced), or a gene whose existence was, prior to over-expression, unknown. The cells can be used to provide desired amounts of a gene product *in vitro* or *in vivo*. The gene product can then be isolated and purified if desired. It can be purified by cell lysis or from the growth medium (as when the vector contains a secretion signal sequence).

The invention also encompasses libraries of cells made by the above described methods. A library can encompass all of the clones from a single transfection experiment or a subset of clones from a single transfection experiment. The subset can over-express the same gene or more than one gene, for example, a class of genes. The transfection can have been done with a single construct or with more than one construct.

A library can also be formed by combining all of the recombinant cells from two or more transfection experiments, by combining one or more subsets of cells from a single transfection experiment or by combining subsets of cells from separate transfection experiments. The resulting library can express the same gene, or more than one gene, for example, a class of genes. Again, in each of these individual transfections, a unique construct or more than one construct can be used.

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Libraries can be formed from the same cell type or different cell types. The invention is also directed to methods for making libraries by selecting various subsets of cells from the same or different transfection experiments.

The invention accordingly is also directed to methods of using libraries of cells to over-express endogenous genes. The library is screened for the expression of the gene and cells are selected that express the desired gene product. The cell can then be used to purify the gene product for subsequent use. Expression in the cell can occur by culturing the cell *in vitro* or by allowing the cell to express the gene *in vivo*.

In preferred embodiments of the invention, the methods include a process wherein the expression product is purified. In highly preferred embodiments, the cells expressing the endogenous gene product are cultured so as to produce amounts of gene product feasible for commercial application, and especially diagnostic and therapeutic and drug discovery uses.

Any of the methods can further comprise introducing double-strand breaks into the genomic DNA in the cell prior to or simultaneously with vector integration.

The invention also encompasses novel vector constructs for activating gene expression or over-expressing a gene through non-homologous recombination. The novel construct lacks homologous targeting sequences. That is, it does not contain nucleotide sequences that target host cell DNA and promote homologous recombination at the target site, causing over-expressing of a cellular gene via the introduced transcriptional regulatory sequence.

Novel vector constructs include a vector containing a transcriptional regulatory sequence operably linked to an unpaired splice donor sequence and further contains an amplifiable marker.

Novel vector constructs include constructs with a transcriptional regulatory sequence operably linked to a translational start codon, a signal secretion sequence, and an unpaired splice donor site; constructs with a transcriptional regulatory sequence, operably linked to a translation start codon,

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The methods are also capable of producing over-expression of known and/or characterized genes for *in vitro* or *in vivo* protein production.

A "known" gene relates to the level of characterization of a gene. The invention allows expression of genes that have been characterized as well as of genes that have not been characterized. Different levels of characterization are possible. These include detailed characterization, such as cloning, DNA, RNA, and/or protein sequencing, and relating the regulation and function of the gene to the cloned sequence (e.g., recognition of promoter and enhancer sequences, functions of the open reading frames, introns, and the like). Characterization can be less detailed, such as having mapped a gene and related function, or having a partial amino acid or nucleotide sequence, or having purified a protein and ascertained a function. Characterization may be minimal, as when a nucleotide or amino acid sequence is known or a protein has been isolated but the function is unknown. Alternatively, a function may be known but the associated protein or nucleotide sequence is not known or is known but is not related to the function. Finally, there may be no characterization in that both the existence of the gene and its function are not known. The invention allows expression of any gene at any of these or other specific degrees of characterization.

Many different proteins can be activated or over-expressed by a single activation construct and in a single set of transfections. Thus, a single cell or different cells in a set of transfectants (library) can over-express more than one protein following transfection with the same or different constructs. Previous activation methods require a unique construct to be created for each gene to be activated.

Further, many different integration sites adjacent to a single gene can be created and tested simultaneously using a single construct. This allows rapid determination of the optimal genomic location of the activation construct for protein expression.

Using previous methods, the 5' end of the gene of interest had to be extensively characterized with respect to sequence and structure. For each

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into the genome of the cell by non-homologous recombination, and allowing over-expression of the endogenous gene in the cell. The method does not require previous knowledge of the sequence of the endogenous gene or even of the existence of the gene. Where the sequence of the gene to be activated is known, however, the constructs can be engineered to contain the proper configuration of vector elements (e.g., location of the start codon, addition of codons present in the first exon of the the endogenous gene, and the proper reading frame) to achieve maximal overexpression and/or the appropriate protein sequence.

The cell containing the vector is screened for expression of the gene.

The cell over-expressing the gene can be cultured *in vitro* so as to produce desired amounts of the gene product of the endogenous gene that has been activated or whose expression has been increased. The gene product can then be isolated and purified to use, for example, in protein therapy or drug discovery.

Alternatively, the cell expressing the desired gene product can be allowed to express the gene product *in vivo*.

The vector construct can consist essentially of the transcriptional regulatory sequence.

Alternatively, the vector construct can consist essentially of the transcriptional regulatory sequence and the amplifiable marker.

The invention, therefore, is also directed to methods for over-expressing an endogenous gene in a cell, comprising introducing a vector containing a transcriptional regulatory sequence and an amplifiable marker into the cell, allowing the vector to integrate into the genome of the cell by non-homologous recombination, and allowing over-expression of the endogenous gene in the cell.

The cell containing the vector is screened for over-expression of the gene.

The cell over-expressing the gene is cultured such that amplification of the endogenous gene is obtained. The cell can then be cultured *in vitro* so as to produce desired amounts of the gene product of the amplified endogenous gene that has been activated or whose expression has been increased. The gene product can then be isolated and purified.

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Alternatively, following amplification, the cell can be allowed to express the endogenous gene and produce desired amounts of the gene product *in vivo*.

The vector construct can consist essentially of the transcriptional regulatory sequence and the splice donor sequence.

The invention, therefore, is also directed to methods for over-expressing an endogenous gene in a cell comprising introducing a vector containing a transcriptional regulatory sequence and an unpaired splice donor sequence into the cell, allowing the vector to integrate into the genome of the cell by non-homologous recombination, and allowing over-expression of the endogenous gene in the cell.

The cell containing the vector is screened for expression of the gene.

The cell over-expressing the gene can be cultured *in vitro* so as to produce desirable amounts of the gene product of the endogenous gene whose expression has been activated or increased. The gene product can then be isolated and purified.

Alternatively, the cell can be allowed to express the desired gene product *in vivo*.

The vector construct can consist essentially of a transcriptional regulatory sequence operably linked to an unpaired splice donor sequence and also containing an amplifiable marker.

Other activation vectors include constructs with a transcriptional regulatory sequence and an exonic sequence containing a start codon; a transcriptional regulatory sequence and an exonic sequence containing a translational start codon and a secretion signal sequence; constructs with a transcriptional regulatory sequence and an exonic sequence containing a translation start codon, and an epitope tag; constructs containing a transcriptional regulatory sequence and an exonic sequence containing a translational start codon, a signal sequence and an epitope tag; constructs containing a transcriptional regulatory sequence and an exonic sequence with a translation start codon, a signal secretion sequence, an epitope tag, and a sequence-specific

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dihydrofolate reductase (DHFR), adenosine deaminase (ada), dihydro-orotase glutamine synthetase (GS), and carbamyl phosphate synthase (CAD).

The vector may contain eukaryotic viral origins of replication useful for gene amplication. These origins may be present in place of, or in conjunction with, an amplifiable marker.

The vector may also contain genetic elements useful for the propagation of the construct in micro-organisms. Examples of useful genetic elements include microbial origins of replication and antibiotic resistance markers.

These vectors, and any of the vectors disclosed herein, and obvious variants recognized by one of ordinary skill in the art, can be used in any of the methods described above to form any of the compositions producible by those methods.

Nonhomologous integration of the construct into the genome of a cell results in the operable linkage between the regulatory elements from the vector and the exons from an endogenous gene. In preferred embodiments, the insertion of the vector regulatory sequences is used to upregulate expression of the endogenous gene. Upregulation of gene expression includes converting a transcriptionally silent gene to a transcriptionally active gene. It also includes enhancement of gene expression for genes that are already transcriptionally active, but produce protein at levels lower than desired. In other embodiments, expression of the endogenous gene may be affected in other ways such as downregulation of expression, creation of an inducible phenotype, or changing the tissue specificity of expression.

Cells produced by this method can be used to produce protein *in vitro* (e.g., for use as a protein therapeutic) or *in vivo* (e.g., for use in cell therapy).

The invention also encompasses cells made by any of the above methods. The invention encompasses cells containing the vector constructs, cells in which the vector constructs have integrated, and cells which are over-expressing desired gene products from an endogenous gene, over-expression being driven by the introduced transcriptional regulatory sequence.

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and/or DNA breaking agents, applied either together (to the same cells) or separately (applied to individual groups of cells and then combining the cells together to produce the library). The library can be composed of multiple types of cells containing a single or multiple constructs which were integrated into the genome of a cell treated with radiation, restriction enzymes, and/or DNA breaking agents, applied either together (to the same cells) or separately (applied to individual groups of cells and then combining the cells together to produce the library).

The invention is also directed to methods for making libraries by selecting various subsets of cells from the same or different transfection experiments. For example, all of the cells expressing nuclear factors (as determined by the presence of nuclear green fluorescent protein in cells transfected with construct 20) can be pooled to create a library of cells with activated nuclear factors. Similarly, cells expressing membrane or secreted proteins can be pooled. Cells can also be grouped by phenotype, for example, growth factor independent growth, growth factor independent proliferation, colony formation, cellular differentiation (e.g., differentiation into a neuronal cell, muscle cell, epitelial cell, etc.), anchorage independent growth, activation of cellular factors (e.g., kinases, transcription factors, nucleases, etc.), gain or loss of cell-cell adhesion, migration, or cellular activation (e.g., resting versus activated T cells).

The invention is also directed to methods of using libraries of cells to over-express an endogenous gene. The library is screened for the expression of the gene and cells are selected that express the desired gene product. The cell can then be used to purify the gene product for subsequent use. Expression of the cell can occur by culturing the cell *in vitro* or by allowing the cell to express the gene *in vivo*.

The invention is also directed to methods of using libraries to identify novel gene and gene products.

The invention is also directed to methods for increasing the efficiency of gene activation by treating the cells with agents that stimulate or effect the